STUDIES ON MUSTARD-STIMULATED PROTEASES AND INHIBITORS IN HUMAN EPIDERMAL KERATINOCYTES (HEK): DEVELOPMENT OF ANTIVESICANT DRUGS

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ABSTRACT

Protease stimulation in HEK due to mustard (sulfur mustard, SM; nitrogen mustard, NM) exposure is well established. However, the specific protease(s) stimulated by mustard and the protease substrates remain to be determined. In this study, we observed that mustard stimulates several proteases, and the epidermal-dermal attachment protein laminin-5 is one of the substrates. In mustard-exposed skin, laminin-5 degradation results in the detachment of the epidermis from the dermis and, therefore, vesication. We utilized gelatin zymography, Western blotting, and immuno-fluorescence staining techniques to study the mustard-stimulated proteases and laminin-5 degradation in HEK. Two major protease bands (64 kDa and 72 kDa) were observed by zymography in mustard-exposed cells. Addition of serine protease inhibitors (50 μM ICD 2812 or 1 mM PMSF), the metalloprotease inhibitor 1, 10-phenanthroline (1 mM), or the caspase inhibitor Z-VAD-FMK (10 μM) to cells prior to SM exposure decreased the mustard-stimulated protease bands (zymography), and completely prevented mustard-induced laminin-5 degradation (Western blotting, immuno-fluorescence). In conclusion, our results in the HEK model indicate that (a) mustard stimulates multiple proteases in the skin, and (b) protease inhibitors are prospective vesicant countermeasures.

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INTRODUCTION

Protease stimulation in epidermal keratinocytes is believed to be one of the mechanisms of vesication (skin blister formation) due to the chemical warfare agent sulfur mustard (SM, bis-(2-chloroethyl) sulfide). However, the specific protease(s) stimulated by mustard and the protease substrates remain to be determined. Smith *et al*. (1) and Mol *et al*. (2) reported mustard stimulation of serine protease and metalloprotease, respectably in skin cells *in vitro*. Mustard-stimulated proteases cause the separation of the epidermis from the dermis by degrading attachment proteins such as laminin-5. Petrali *et al.* (Petrali, J. P., USAMRICD, APG, MD, personal communication) reported that laminin-5 is affected during pathologies associate with SM exposure. Therefore, in this study, we utilized gelatin zymography, Western blotting, and immuno-fluorescence staining techniques to study the mustard (SM, nitrogen mustard (NM)) stimulated proteases and laminin-5 degradation in HEK. The purpose of this research was to obtain new knowledge regarding the specific mustard-stimulated proteases, their functions, and inhibitors, so that a protease inhibitor-based antivesicant approach could be developed.

MATERIALS AND METHODS

Materials: HEK, human keratinocyte growth supplement were purchased from Cascade Biologics. Anti-laminin-5 $\alpha 3$, $\beta 3$, $\gamma 2$ polycolonal antibodies were from Santa Cruz. Chromozym TRY (serine specific substrate), 1, 10- phenanthroline, PMSF, and E-64 were from Roche. Coomassie blue R-250 was from Sigma.

Cell culture: Sulfur mustard and nitrogen mustard treatment-NHEK cultures were initiated in basal media from frozen stock (passage 2) using 0.2×10^6 cells per 75 cm^2 plastic tissue culture flaks. Approximately 80% confluent cultures were subcultured to passage 3 to be used in the experiments. Exposure of cell with 300 μM sulfur mustard was done at USAMRICD, APG, MD.

Zymography: Electrophoretic gelatin zymography was conducted as described by Heussen and Dowdle (3). Cell lysates were prepared at 16 hours after mustard exposure and in Mammalian Protein Extraction Reagent from Pierce. Protein concentration was determined by the BCA™ protein assay described by Pierce. Cell lysates were normalized to equal protein concentrations. A 50 μg aliquot of each sample was lyophilized and mixed with 20 μl Tris-Glycine SDS sample buffer (2X) in the absence of any reducing agent, and incubated for 10 minutes at room temperature. The samples were electrophoresed on 10% polyacrylamide gels co-polymerized with 1 mg/ml gelatin (Invitrogen). After electrophoresis, the gels were incubated in zymogram renaturing buffer (Invitrogen) containing 2.5% Triton x100 with gentle agitation for 30 minutes at room temperature and then incubate in zymogram developing buffer (Invitrogen) for 30 minutes followed by replacing with fresh zymogram developing buffer and incubation at 37^0C overnight. After incubation, the gels were stained with 0.5% Coomassie Brilliant Blue R-250 (Sigma) and de-stained with de-staining solution (40% methanol, 10% acetic acid in distilled water). Protease activity was detected as a clear band against a dark blue background of Coomassie-Blue R-250 stained gelatin. For inhibition studies, one of 1 mM of 1, 10 phenanthroline, 1 mM of PMSF, 10 μM of Z-VAD-FMK, and 100 μM of E-64 were added to flasks followed by 300μM sulfur mustard treatment. Elution of gel band was done by using the Bio-Rad Model 422 electroeluter.

Chromozym TRY assay: Protease activity in electroeluted samples was assayed according to Cowan et al. (4) using chromozym TRY substrate.

Western blot analysis: HEK lysate or medium was used and Western blotting was done using laminin-5 $\gamma 2$ ploYclonal antibody.

Immunofluorescence staining: Lamini-5 distribution and degradation were determined by an immunofluorescence staining using laminin-5 γ2 primary antibody. Rhodamine-conjugated secondary antibody for laminin-5 γ2 for 1 hour at room temperature and then washed with PBS 3 times at 5 minutes intervals. Slides were mounted with mounting solution (SIGMA).

RESULTS AND DISCUSSION

Protease bands in zymogram analysis: Gelatin zymogram of the unexposed HEK control group demonstrated that small amounts of protease were present, mainly as 72 kDa and 64 kDa. We observed an increase in 72 kDa and 64 kDa protease band due to mustard *(figure 1)*. The 72 kDa band from SM treated cell extract was cut and electroeluted. The 72 kDa band showed increased SM stimulated protease activity by the Chromozym TRY method compared to untreated control *(figure 2)*.

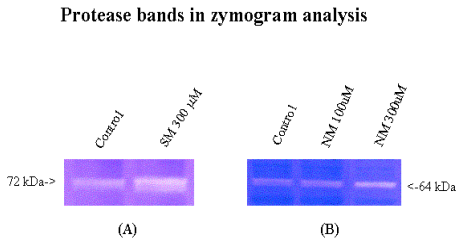

Figure 1. Gelatin zymogram of unexposed HEK control group demonstrated that small amounts of proteases were present, mainly 72kDa and 64 kDa. An increase in 72 kDa (A) and 64 kDa (B) proteases bands was observed due to mustard exposure.

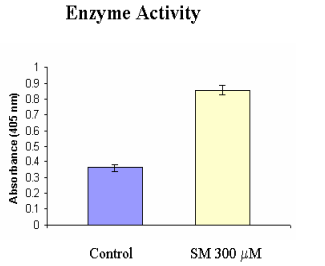

Figure 2. The 72 kDa band was cut from gelatin zymogram gel and eluted. The band showed increased SM stimulated protease activity by the Chromozym TRY assay method compared to untreated control.

Protease inhibitor study: Addition of the serine protease inhibitor ICD 2812 (50 μM), phenylmethylsulfinyl fluoride (PMSF, 1mM), and pan-caspase inhibitor Z-VAD-FMK (10 μM) to HEK prior to SM exposure decreased the SM stimulated 72 kDa band as detected by zymography. Addition of metalloprotease inhibitor 1, 10-phenanthroline (1 mM), decreased the 64 kDa protease band in SM-exposed HEK *(figure 3)*. The cysteine protease inhibitor E-64 (100 μM) was ineffective.

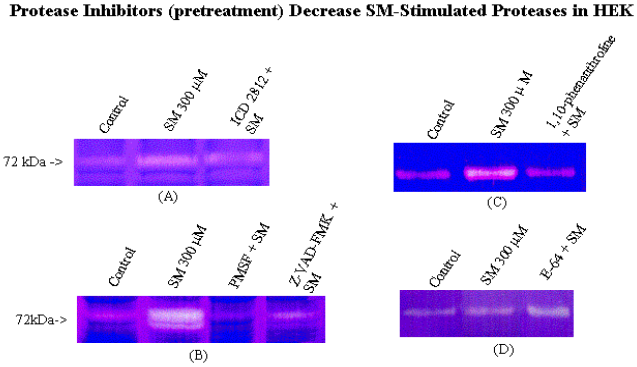

Figure 3. Addition of a serine protease inhibitor ICD 2812 (50 μM) (A), PMSF (1mM) or pan-caspase inhibitor Z-VAD-FMK (10 μM) (B) to HEK prior to SM-exposure decreased the SM stimulated 72 kDa band as detected by zymography. Addition of metalloprotease inhibitor 1, 10 phenanthroline, decreased the 64 kDa protease band in SM-exposed HEK (C). The cysteine protease inhibitor E-64 (100 μM) was ineffective (D).

Protection of SM-induced laminin-5 degradation by protease inhibitors: Laminin-5 degradation was detected with 300 μM sulfur mustard and 300 μM nitrogen mustard treatment in HEK. The protection of laminin-5 degradation was found with addition of the serine protease inhibitor PMSF, or pan-caspase inhibitor Z-VAD-FMK. The metalloprotease inhibitor 1, 10-phenanthroline protected against laminin-5 degradation due to 300 μM SM (16 hour) *(figure 4)*. Immuno-fluorescence staining with anti-laminin–5 γ2 antibody revealed degradation of laminin-5 γ2 with 300 μM nitrogen mustard and the degradation of laminin–5 γ2 due to 300 μM nitrogen mustard was protected with the serine protease inhibitor PMSF and the metalloprotease inhibitor 1, 10-phenanthroline. Addition of the pan-caspase inhibitor Z-VAD-FMK also protected the degradation of laminin-5 γ2 *(figure 5)*. However, the cysteine protease inhibitor E-64 (100 μM) did not protect against degradation of laminin-5 γ2.

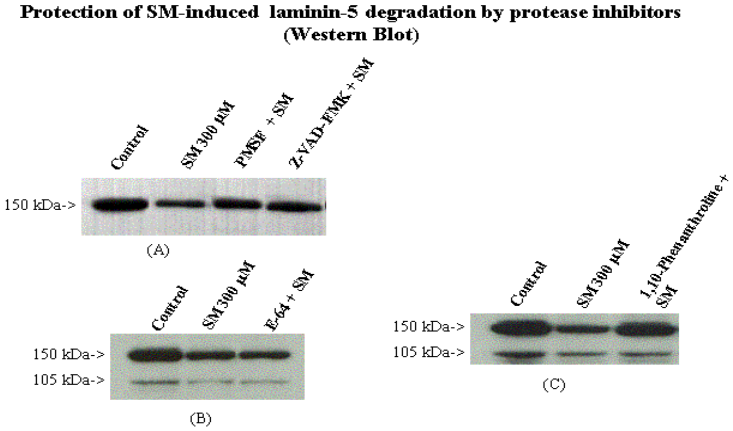

Figure 4. The protection of laminin-5 degradation was detected with addition of the serine protease inhibitor PMSF (1mM), pan-caspase inhibitor Z-VAD-FMK (10 μM) (A) and metalloprotease inhibitor 1,10-phenanthroline (1mM) (C), but no protection was detected with E-64 (100 μM) (B) treatment before SM-exposure in HEK.

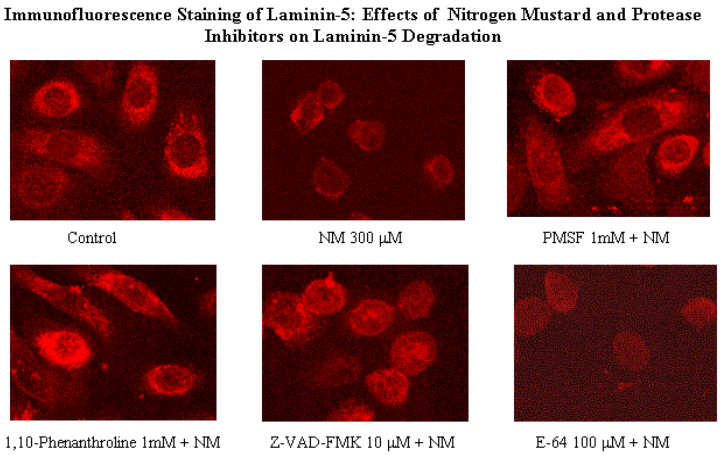

Figure 5. Immuno-fluorescence staining with anti-laminin-5 γ2 antibody revealed that degradation of laminin-5 γ2 by NM (300 μM) treatment and the degradation of laminin-5 γ2 was prevented with treatment of PMSF, 1,10-phenanthroline, or the pan-caspase inhibitor Z-VAD-FMK, but not cysteine protease inhibitor E-64.

In this study, three types of proteases induced by SM in HEK have been identified and characterized. Further analysis will be required to determine their physiological and pathological functions.

CONCLUSION

SM stimulates multiple types of proteases: Serine protease, metalloprotease, and caspases. SM stimulated protease degrades laminin-5, an epidermal-dermal attachment protein relevant to vesication. SM-induced laminin-5 degradation is partially prevented by pretreatment of HEK with inhibitors of serine protease (PMSF), metalloprotease (1, 10-phenanthroline), and caspases (Z-VAD-FMK), but not the prototype cysteine protease inhibitor (E-64). These results suggest a role of apoptosis in SM-induced laminin-5 degradation and, therefore, vesication. These results also suggest that protease inhibitors may be prospective antivesicant drugs.

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